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Deficiency of Peptidoglycan and Lipopolysaccharide Components in *Rickettsia tsutsugamushi*

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Analyses of chemical composition in whole cells of *Rickettsia tsutsugamushi* were performed and compared with those of the other rickettsiae and gram-negative bacteria. The results indicated that *R. tsutsugamushi* does not contain detectable amounts of 3-deoxy-D-mannooctulosonic acid, heptose, muramic acid, or glucosamine (<2, <2, <3, and <3 nmol/mg, respectively). The microorganism was found to contain four kinds of fatty acids (16:0, 18:0, 18:1, and 18:2), but not hydroxy fatty acids. Furthermore, in analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver or Coomassie blue staining, lipopolysaccharide bands were not detected in preparations treated with proteinase K. It is concluded that *R. tsutsugamushi* has little or no peptidoglycan or lipopolysaccharide.

The pathogenic agent of scrub typhus fever, *Rickettsia tsutsugamushi*, has a cell envelope similar to that of gram-negative bacteria, i.e., an outer membrane and a plasmic membrane (20, 23) with a dense intermediate layer of peptidoglycan (PG) that cannot be distinguished clearly. In 1960, Allison and Perkins (1) reported the presence of muramic acid in *Coxiella burnetii* and *Rickettsia typhi*, and Myers et al. (15) found diaminopimelic acid in *Rickettsia prowazekii*, *R. typhi*, *Rochalimaea quintana*, and *C. burnetii*. Recently, Amano et al. (3, 5) reported the structure of PG of *C. burnetii*. Furthermore, the presence of lipopolysaccharides (LPS) in rickettsiae except for *R. tsutsugamushi* was described by several investigators (4, 6, 16, 17). On *R. tsutsugamushi*, however, there are no reports of its chemical composition, especially of the envelope, and this led us to the chemical analysis of this microorganism to determine the presence or absence of PG and LPS. In this communication, we describe some evidence of the absence of both components in *R. tsutsugamushi* on the basis of chemical analysis using various techniques including sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

R. tsutsugamushi Gilliam was propagated in suspension cultures of L cells and purified by Percoll density gradient centrifugation as described by Tamura et al. (20). Rickettsia prepared by the same method were used in our previous studies (14, 19, 22, 26), in which we recognized that each separate preparation showed relatively constant polypeptide patterns in repeated SDS-PAGE analyses, as well as minor reactivity with anti-L-cell homogenate antiserum, suggesting that the preparations do not contain notable amounts of contaminants originating from host cells. *R. typhi* Wilmington, *R. prowazekii* E, and *C. burnetii* Nine Mile, phase I, were propagated and purified by Renografin density gradient centrifugation (3–5). *Salmonella typhimurium* LT2 and *Proteus vulgaris* OX19 were cultivated in a liquid medium as described previously (2). Amino acids and amino sugars were analyzed in a Shibata AA-100 (Shibata Scientific Technology, Ltd., Tokyo, Japan) or K202 (Kyowa Seimitsu, Ltd., Tokyo, Japan) amino acid autoanalyzer by using 1 mg (dry weight) of sample hydrolyzed in 4 N HCl at 100°C for 15 h in a sealed glass ampoule (5). Contents of protein and total phosphorus were determined by the method of Lowry et al. (12, 13). Total amounts of neutral sugars were measured by the phenol-sulfuric acid method (7) with glucose as a reference standard. 3-Deoxy-D-mannooctulosonic acid (KDO) was analyzed by the method of Karkhanis et al. (10). Fatty acids were analyzed as methyl esters in a Hitachi 163 gas chromatograph on a Chromosorb WAW column containing 15% ethylene glycol succinate (Gasukuro Kogyo, Ltd., Tokyo, Japan) as described previously (5). Identification of neutral sugars and their quantitative analysis were carried out with the sugars as alditol acetates in a Hitachi 163 gas chromatograph on a 3% ECNSS-M Gas-Chrom Q glass column as described previously (4). Gels for SDS-PAGE were prepared by the modified methods of Laemmli (11) as described previously (19), except the stacking gel and separation gel contained 5 and 12.5% of polyacrylamide, respectively, and were used for protein and LPS analyses. Each sample for SDS-PAGE was boiled for 5 min in sample buffer and immediately applied to each slot. After electrophoresis, the gels were fixed with an aqueous solution of 25% isopropyl alcohol and 7% acetic acid and stained with Coomassie brilliant blue or by the two silver staining methods (one stains both LPS and proteins and the other stains only LPS) described by Hitchcock and Brown (9). Some samples for SDS-PAGE were pretreated with proteinase K (PK; Sigma Chemical Co., St. Louis, Mo.) as described by Hitchcock and Brown (9).

On the basis of comparative chemical analyses of four rickettsiae, including *R. tsutsugamushi*, and two gram-negative bacteria (Table 1), the protein contents in the whole-cell (WC) preparations of the microorganisms were not significantly different from each other (48 to 57%), while the content of neutral sugar in *R. tsutsugamushi* was considerably higher than those in the other microorganisms. Gas chromatography of alditol acetate derivatives of an acid hydrolysate of *R. tsutsugamushi* appeared in one peak at a position corresponding to the retention time of the alditol acetate derivative of ribose. This ribose may be derived from the nucleic acids. Heptose and KDO were barely detectable

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TABLE 1. Chemical analyses of rickettsial and other bacterial WC[a]

Microorganism	Protein (%)	Neutral sugar (nmol/mg)	Total P (nmol/mg)	Heptose (nmol/mg)	KDO (nmol/mg)	Fatty acid (%)
R. tsutsugamushi	53	706	330	<2	<2	2.3
R. typhi	54	294	1,992	24	48	ND[b]
R. prowazekii	50	333	1,435	30	40	ND
C. burnetii	53	527	738	48	26	ND
S. typhimurium	57	337	498	35	19	ND
P. vulgaris	48	147	270	22	14	ND

[a] The data on *R. tsutsugamushi* are the average of double analyses of two different preparations; the other data are the average of double analyses of one preparation.

[b] ND, Not determined.

in the *R. tsutsugamushi* WC, while the other rickettsiae and bacteria contained both sugars (22 to 48 and 14 to 48 nmol/mg, respectively). Because these sugars are structural components of the bacterial LPS, the data suggest the lack of LPS in *R. tsutsugamushi*.

The contents of muramic acid and glucosamine in each microorganism were determined with an amino acid analyzer. The former sugar is a constituent of PG, and the latter is a constituent of PG and LPS. *R. typhi* and *C. burnetii* contained both muramic acid (25 and 28 nmol/mg, respectively) and glucosamine (48 and 56 nmol/mg, respectively), suggesting that both rickettsiae contain PG and LPS as previously reported by Amano et al. (5). The WC of *S. typhimurium* and *P. vulgaris*, which are known to have PG and LPS, contained 50 and 70 nmol of glucosamine per mg, respectively. However, the muramic acid and glucosamine contents of *R. tsutsugamushi* WC were less than 3 nmol/mg in double analyses of each of two independent purified samples, indicating that *R. tsutsugamushi* possesses no more than one-eighth the amount of PG and LPS found in other rickettsiae. Gas chromatographic analysis of the fatty acids in *R. tsutsugamushi* showed that this rickettsia contained two saturated linear fatty acids (16:0 and 18:0; 25 and 27%, respectively) and two unsaturated linear fatty acids (18:1 and 18:2; 44 and 4%, respectively); however, hydroxy fatty acids were not detected. Generally, LPS contains hydroxy fatty acids as constituents of lipid A, and the results obtained support the absence of LPS in *R. tsutsugamushi*. The presence of hydroxymyristic acid has been detected in *R. typhi*, *R. prowazekii*, *Rickettsia canada*, *Rickettsia akari*, and *Rickettsia rickettsii* (21). However, the LPS of *C. burnetii* has no hydroxy fatty acid and a very low content of glucosamine, but does contain detectable amounts of KDO and heptose (4). To confirm the absence of LPS in *R. tsutsugamushi*, boiled samples of the rickettsial WC in sample buffer were treated with PK at 60°C for 60 min and analyzed by SDS-PAGE. After silver staining for both LPS and proteins, the sample without PK treatment showed many bands distributed from low- to high-molecular-weight areas (Fig. 1, lanes 3 and 4). The sample treated with PK revealed only one broad band of less than 12.4 kilodaltons, which was not detected in the samples without PK treatment (Fig. 1, lanes 1 and 2). This low-molecular-weight band was not stained by silver staining specific to LPS (9), but was stained with Coomassie blue, suggesting that this band may be a crowd of polypeptides hydrolyzed with the PK (data not shown).

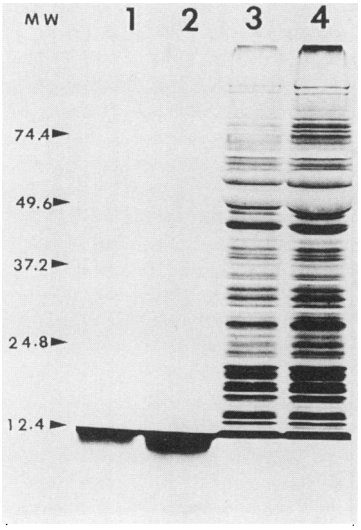

FIG. 1. Comparison of *R. tsutsugamushi* WC, untreated or treated with PK, by SDS-PAGE. The gel was stained by the silver staining technique for both LPS and proteins. Lanes: 1 and 2, PK-treated WC (50 and 100 μg, respectively); 3 and 4, PK-untreated WC (50 and 100 μg, respectively). Molecular weight markers (Oriental Yeast Co., Tokyo, Japan) (kilodaltons): cytochrome *c* oligomer; monomer, 12.4; dimer, 24.8; trimer, 37.2; tetramer, 49.6; hexamer, 74.4.

Muramic acid, glucosamine, heptose, KDO, and hydroxy fatty acid are the constituents of bacterial PG and LPS, and the deficiency of these components in *R. tsutsugamushi* WC indicates that the rickettsia does not contain PG and LPS or that the PG and LPS of this species may be composed of chemical constituents different from those of the other microorganisms.

R. tsutsugamushi is very fragile and easily disrupted by light sonication or by osmotic shock (20). The multiplication of *R. tsutsugamushi* is not affected at all by penicillin, which is known as an inhibitor of bacterial PG synthesis, whereas this antibiotic disturbs the growth of other rickettsiae such as *R. prowazekii* and *R. rickettsii* (24, 25). Additionally, treatment of *R. tsutsugamushi* with periodate does not alter its antigenic properties in the neutralization test of infectivity by immune sera (8). These observations on *R. tsutsugamushi* may all easily be understandable if the microorganism does not have PG and LPS. The data reported here suggest that the composition of the cell envelope of *R. tsutsugamushi* differs from that of the other rickettsiae and may give us an important criterion in the taxonomy of rickettsiae. Morphological differences between the cell envelopes of *R. tsutsugamushi* and the other species of rickettsiae (18) also support the idea of the different constitution of cell envelopes among these rickettsiae.

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